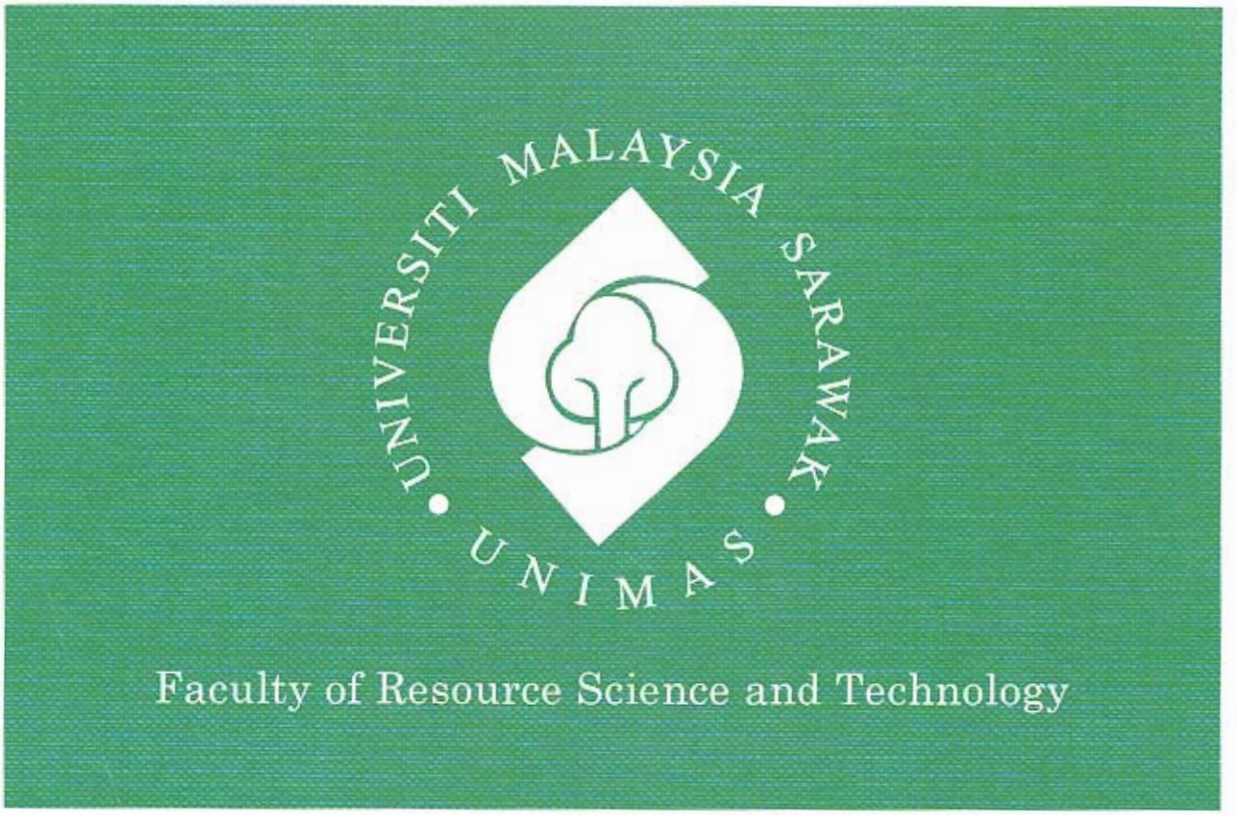

ANTIMICROBIAL ASSAY OF TRIMETHOXY CARBONYL THIOUREA COMPLEXES

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Bachelor of Science with Honours
(Resource Biotechnology)
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ANTIMICROBIAL ASSAY OF TRIMETHOXY CARBONYL THIOUREA COMPLEXES

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This project is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honour in Resource Biotechnology.

Programme of Resource Biotechnology
Department of Molecular Biology

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DECLARATION

I, Umme Najehah binti Ahmad, hereby declare that this final year project report is done by me. I had put 80% effort in finishing the final year project and report writing, with 10% contributed by my supervisor in guiding, checking, and correcting my experiment and also the writing. The remaining 10% contributions are from the postgraduate students and my fellow course mate who had helped me throughout the project.

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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LIST OF ABBREVIATIONS

DMSO	Dimethyl Sulfoxide
HIV	Human Immunodeficiency Virus
MHA	Mueller-Hinton Agar
PDA	Potato Dextrose Agar
OD	Optical Density
EUCAST	European Committee on Antimicrobial Susceptibility Testing
µg/ml	micro gram per milliliter
ppm	parts per million
mm	millimeter
nm	nanometer
°C	Degree Celsius

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Antimicrobial Assay of Trimethoxy Carbonyl Thiourea Complexes

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ABSTRACT

Thiourea complexes had been proven to have the antimicrobial activity against specific bacteria and fungi. Seven series of different thiourea complexes compound were newly synthesized by the Department of Chemistry, UNIMAS and being tested in order to determine its antimicrobial activity on six different bacteria from Gram positive (*Staphylococcus aureus*, *Listeria monocytogenes*, *and Bacillus cereus*) and Gram negative bacteria (*Escherichia coli*, *Enterobacter aerogenes*, and *Salmonella entriditis*) and three species of fungi (*Aspergillus flavus*, *Aspergillus niger*, and *Trichomonas vaginalis*). The antimicrobial assay for bacteria was determined by using the Disc Diffusion technique and Antifungal testing for the antifungal assay. From the experiment, it was found that all the thiourea series had shown the resistance ability towards the tested bacteria, from good to moderate antibacterial activity while for the antifungal assay, all series showed resistance towards *Aspergillus flavus* and *Aspergillus niger* but no towards *Tricoderma viride*. This indicated that all thiourea complexes does not exhibit good antifungal ability towards *Trichoderma viridae* compare to the other test fungi.

Key words: Thiourea siries, antimicrobial assay, antifungal assay

ABSTRAK

Kompleks kepada thiourea telah terbukti mengandungi antimikrobial aktiviti dalam melawan sesetengah bakteria dan kulat. Tujuh siri kompleks thiourea yang berbeza-beza, yang mana baru dihasilkan oleh Jabatan Kimia, UNIMAS dan telah diuji untuk mengkaji dan menentukan aktiviti antimikrobial nya keatas enam jenis bakteria yang berlainan daripada bakteria Gram positif (Staphylococcus aureus, Listeria monocytogenes, dan Bacillus cereus), bakteria Gram negatif (Escherichia coli, Enterobacter aerogenes, dan Salmonella entriditis) dan juga tiga spesis fungi (Aspergillus flavus, Aspergillus niger, and Trichoderma viridae). Untuk pengasaian antimikrobial, kaedah yang telah digunakan untuk bakteria ialah kaedah Disk Resapan manakala kaedah Antikulat telah digunakan untuk pengasaian antikulat. Daripada eksperimen yang telah dijalankan, kesemua siri thiourea menunjukkan keupayaan melawan kepada bakteria, dengan menunjukkan aktiviti antibakterial daripada tahap yang bagus kepada sederhana manakala untuk pengasaian antikulat, kesemua siri thiourea telah menunjukkan keupayaan melawan kepada A. flavus dan A. niger tetapi tidak kepada T. viridae. Ini menunjukkan kesemua kompleks thiourea tidak mempamerkan kebolehan antikulat yang bagus kepada T. viridae berbanding dengan fungi yang lain yang telah diuji.

Kata kunci: *Siri thiourea, pengasaian antimikrobial, pengasaian antikulat.*

1.0 Introduction

In the effort to discover the new antibiotic that posses a good potential of antimicrobial activity, scientists have focused upon the other alternative way of producing antibiotic upon relying on extract from the natural resources. There are many examples of the antibiotics from natural resources. For example, the penicillin which is secreted from the fungus of *Penicillium chrysogenum* species. The natural of *Penicillium chrysogenum* species secreted the penicillin as a chemical is actually to defense themselves againts other bacterium in the ecosystem, such as the soil ecosystem (John, 2010). Apart from fungus, another natural resources that has the antibiotic properties is garlic (Bardot, J. B., 2012). This traditional herb has been used worldwide for thousands of years for the medicinal purposes . It was said that it can treat many bacteria infectious such as *Helicobacter pylori* (Bardot, J. B., 2012). Because of it's antioxidant elements and active ingredients called allicin, it has the antibacteria, antivirus and also the antifungal ability.

Although natural antibiotics has been used widely and proven to be effective, it do has disadvantages over synthesized compound. The process of isolating the natural antibiotics involve many, complicated processes and low production yield. The penicillin production for instance, need to undergoes the fermentation process and only will produced the penicillin in the second stage of the batch fermentation (O'Hare *et al.*, 2012). As for the antibiotic synthesis, the synthesis process may not be as difficult as the natural antibiotics. Furthermore, bulk production of antibiotic is possible by antibiotic synthesis. In addition, synthesis antibiotic also has the advantage in terms there is the freedom to design the chemical structure to fit the target microbes. A few examples of synthesis antibiotic commercially available are polyether, antracycline and tetracyline (Smelcerovic *et al.*, 2000).

Thiourea derivative had been reported to have active compound including antiviral, antibacterial, and antifungus, and antiherbicidal (Arslan *et al.*, 2009). Some has been used in the treatment of antibacterial and antifungal activity using the different types of bacterial according to the strain which are Gram positive and Gram negative. Based on the report from Arslan *et al.*, (2009) they did use the Gram negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa,* and *Enterobacter cloacae*, from the Gram positive bacteria such as *Enterococcus faecalis, Streptococcus pyogenes,* and *Bacillus cereus*. Meanwhile for the yeast, it was tested on *Candida albicans, Candida krusei,* and *Candida glabrata*. (Arslan *et al.*, 2009). There were many chemical compound within the thiourea had been used in the antimicrobial assay, but none with the Trimethoxy Carbonyl Thiourea.

Trimethoxy Carbonyl Thiourea is a chemical compound that is newly synthesize chemical compound. It is a compound that contains many elements such as methoxy, carbony and of course the thiourea. Thiourea elements posses the special complexes such as the Nickel(Ni^{2+}) and Copper(Cu^{2+}) from the synthesis process. Thiourea derivatives and certain complexes are well known in microbiology field that have special ability as the antimicrobial agent that can resist the growth of bacteria and fungi. This compound usually being used in the antimicrobial assay test in order to determine the antibacterial or antifungal activity of this compound.

Therefore, the objective of this study was:

1) To determine the antimicrobial activity of newly synthesized Trimethoxy Carbonyl Thiourea compounds obtained from Department of Chemistry, Faculty of Resources Science and Technology on the test bacteria and fungi.

2.0 Literature Review

In the field of microbiology, the antimicrobial assay is the common test that is used to determine the effectiveness of the new antimicrobial agent to inhibit the growth of the bacteria or the fungi. The antimicrobial agents must be effective against the selected bacteria and also the fungi. According to (Gulkok *et al.*, 2011) antimicrobial drugs (antibacterial and antifungal) have been successfully used in treatment of infectious diseases cause by the bacteria and fungi since the first half of the 20th century and has been effective in prolonging of the average life expentancy by reducing the deaths caused by infectious diseases.

2.1 The Discovery of Antimicrobial Agent

Nowadays, there are many dangerous disease has been developed from the many kind of bacteria, fungus and also virus. The treatment for these disease may be different as the bacteria and others had been differently developed or mutated, and as for the certain antimicrobial agents it has no longer able to inhibit them from growth. The increasing and misuse of antimicrobial drugs have led to the development of more resistant pathogens to the commonly used antimicrobials (Gulkok *et al.*, 2011). There are many sources for the antimicrobial agents available. The antimicrobial agents are originally obtained from the natural resources and also can be synthesized from certain compound. As the example from the natural resources, the famous penicillin antibiotic has widely used all around the world. It also the earliest discovered antibiotics by the Alexander Flaming in 1982 and derived from the *Penicillium* mold (Bellis, M., 2012). The antibiotic had being released by the *Penicillium* fungi into their environments by means on inhibiting the other organisms as their own defense. At that time, Alexander Flaming observed that the colonies of the bacterium

Staphylococcus aureus were being destroyed by the blue-green mold of *Penicillium notatum* and the further investigation had been done by Howard Florey and Ernst Chain in 1940s (Bellis, M., 2012). The result announced from them proved that the penicillin have the antibacterial agent that could kill certain types of disease-causing bacteria inside the body of human.

For the antimicrobial agent that is synthesized from the chemical compound, the sulfa drugs are the best example. Sulfanilamide was the first antibacterial agent that was discovered (John, 2010). After the discovery of the sulfanilamide, other sulfa drugs such as sulfamethoxazole also had been used as the antimicrobial agents (John, 2010). Sulfanilamide has the ability to inhibit the bacteria by its sulfa drugs elements and the folic acid analogs. Both bacteria and their host human require folic acid for the nucleic acid synthesis and for the protein synthesis, however, bacteria using the folic acid with para-aminobenzoic acid (PABA) while we have to ingest our folic acid. The sulfa drugs are analogs to the PABA and the synthesis of folic acid by the bacteria will be stopping (John, 2010). Therefore automatically, the bacteria will no longer able to synthesized folic acid for their requirements and will die.

Even though the natural extracted antibiotics has been proven to be effective, the discovery of the new drugs by antibiotics synthesis is necessary to provide alternative treatment of the emerging infectious diseases.

2.2 Trimethoxy Carbonyl Thiourea

Trimethoxy Carbonyl Thiourea is a newly produced compound that is containing the carbonyl and thiourea elements. According to Arslan *et al.*, (2009) compound that containing carbonyl groups occupies an important position among organic reagents as the potential donor ligands for transition metal ions. The thiourea elements and their complexes are very versatile ligands which are able to coordinate to a range of metal centres as the neutral ligands, monoions, and diaions (Arslan *et al.*, 2009). Both of the ligands and their metal complexes have the special ability which is the biological activity including antibacterial, antifungal, insecticidal, and herbicidal (Arslan *et al.*, 2009). The chemical will be synthesized earlier in order to get the particular derivatives and also the metal complexes. In an antimicrobial experiment done by Gulkok *et al.*, (2011), they also stated that some urea and thiourea derivatives are already known to be associated with effectiveness of biological activities such as analgesic, antitumor, anti HIV, and certainly antimicrobial properties.

According to the research done by the Arslan *et al.*, (2009), their team also use chemical that have the thiourea derivatives and their Nickel (Ni^{2+}) and Copper (Cu^{2+}) complexes as the antimicrobial agent against certain species of bacteria from different strain and yeast. They used five thiourea derivative ligands, for example (*N*-diethycarbamothioyl) cyclohexanecarboxamide and *N*-(di-*n*-propylcarbamothioyl) cyclohexanecarboxamide against the bacteria from different strain and yeast such as *Staphylococcus aureus, Escherichia coli* and *Candida albicans.*

In the year 2000, novel thiourea compound also had been reported having the role as dual-function of microbicides in against the Human Immunodeficiency Virus (HIV), the most dangerous and fastest growing virus. According to the D'Cruz *et al.*, (2000), among 30 thiourea compounds that they rationally designed and synthesized in order to against the HIV, they found about nine of the thiourea derivatives successfully inhibited both anti-HIV and spermidicidal activity (D'Cruz *et al.*, 2000). The chemical they use such as Phenyl ring-containing thioureas, able to inhibit potent HIV activity with range of usage less than 1 nm and the spermidicidal activity with the range less than 1-9 nM (D'Cruz *et al.*, 2000).

The antimicrobial agent is not limited to thiourea derivatives only. There are also drugs and other chemicals that had been used as the antimicrobial agenst such as pyrazine which is containing the thiazolines and thiozalidinones elements. According to Bonde and Gaikwad, (2004), they use pyrazine in order to fight against the *Mycobacterium tuberculosis* in their in-vitro studies and they found that the compound did show the anti-microbial activity to resist the growth of that bacterium (Bonde and Gaikwad, 2004).

The compound has the carbonyl, thiourea and also the metal complexes which can be use as the antimicrobial agents. Thiourea is well known as the chemical compound that is important industrial field such as in production of textile and dyeing auxiliaries, leaching of ores, pharmaceuticals and pesticides (Ziegler *et al.*, 2003). Thiourea also can be defined as a photographic fixative used in manufacturing of resins and many of its derivatives are anti-thyroid (Reference.MD, 2012). Most of thiourea derivatives also had been successfully used

in the extraction of Cu(II), Ni(II), Co(II), and Pd(II). These reagents are important for the membrane system for selective transport of relevant metals (Arslan, Florke, and Kulcu, 2004).

In other study by Arslan *et al.*, (2009) five different thiourea derivatives and metal complexes that they had used in screening the antimicrobial assay with the bacteria from different strain and also few species of yeast did showed the anti-bacterial and anti-yeast activity. The thiuorea they used successfully inhibit the growth of the bacteria and yeast that they select (Arslan *et al.*, 2009). For the MIC value, the compound did inhibit the bacteria activity within the range between 50 and 400 $\mu g/cm^3$ and for the yeast activity range between 25 and 100 $\mu g/cm^3$.

2.3 Antimicrobial Assay

There are several antimicrobial assay method that are commonly performed by the clinical microbiology laboratories such as disc diffusion and broth dilution method (Barry *et al.*, 1999). The broth microdilution and disc dilution has been applied in many antibacterial and antifungal studies (Arslan *et al.*, 2009, Gosh *et al.*, 2008). According to the report by the Ravi *et al.*, (2010), they found that disc diffusion method was easier to perform, rapid and cost effective. It also has been demonstrated that disc diffusion method is more useful as compared to other methods for antifungal drug susceptibility testing. According to Serrino *et al.*, (2004), they showed the advantage of disc diffusion method over other methods such as broth dilution or E test for voriconazole susceptibility testing of *Aspergillus*. European Committee On Antimicrobial Susceptibility Testing (EUCAST), (1999), also stated that disc diffusion method was preferable method because the method is the oldest approaches to antimicrobial

susceptibility and remains one of the most widely used methods in the routine clinical laboratories (EUCAST,1999). Although broth dilution method is recommended as the standard method by NCCLS, but this method was said associated with some technical drawbacks such as cumber some procedure, time consuming, and technically demanding poor end point precision especially when fungistatic agents are tested. It also associated with the high standard and difficulty of preparation of hyphal inoculums (Ravi *et al.*, 2010).

3.0 Materials and Methods

3.1 Materials

Seven series of Trimethoxy Carbonyl Thiourea complexes (Toulidine+Ni complex, Toulidine+Cu complex, Aniline+Cu, Toulidine, Anisidine, Aniline, and Aniline+Cu) were provided by the Department of Chemistry, Faculty of Resource Science and Technology (FRST). All of the compounds were tested on three Gram positive bacteria, *Enterobacter aerogenes, Staphylococcus aureus,* and *Listeria monocytogenes* and three Gram negative bacteria, *Escherichia coli, Bacillus cereus* and *Salmonella enteriditis* which were obtained from Virology Laboratory. The fungi used in this study were *Aspergillus niger*, *Aspergillus flavus* and *Trichoderma viridae* were obtained from the collection of FRST, UNIMAS.

3.2 Preparation of Media

3.2.1 Mueller-Hinton Agar (MHA)

A total of 21.0 g of MHA powder was dissolved in 1000 ml of distilled water. The mixture was heated to boil with frequent agitation. The media then was autoclaved at 121 °C for 15-20 minutes. The sterilized MHA was poured into petri dishes and left at room temperature to solidify.

3.2.2 Potato Dextrose Agar (PDA)

About 39.0 g of the PDA powder was suspended with 1000 ml distilled water and autoclaved for 15 minutes at 121°C. The sterilized PDA was poured onto the petri dishes and being left at room temperature to solidify.

3.2.3 Preparation of Chemical Compound and Tested Bacteria

For the Dics diffusion method, the stock solutions of all tested compounds were prepared by dissolving 10.0 µg of respective chemical compound of different series in 1 ml of DMSO. Then, stock solution were prepared by 2-fold serial dilution in order to obtain the required concentrations which were 10.0, 5.0, 2.5, 1.25, and 0.625 µg/ml. Before swabbing all the bacteria on the petri dish, the concentration of each of the bacteria were ensured to be the same by using spectrophotometer at 620 nm of wavelegth and 0.6 optical density (Marutescu *et al.*, 2011)

For the antifungal test, the stock solutions were prepared from respective Trimethoxy Carbonyl Thiourea series compound. Each series were diluted about 0.01 g in 1 ml of DMSO in order to get the concentration of 10,000 ppm within certain calculation done. About 50, 80, and 100 µl from the stock solution will appropriately incorporated into the molten PDA medium which were equivalent for 50 ppm,80 ppm, and 100 ppm as the tested concentration around 45 °C in aseptic condition.

3.3 Disc Diffusion method for Antibacterial Activity

This method was performed based on Gosh *et al.*, (2008). By using the sterilized cotton swab, it was dipped in the respective cultures and will be swabbed on MHA. The sterilized disc paper of 5 mm diameter were placed onto the MHA and 10 µl of the chemical compound with different concentration were loaded on the disc. Two positive control, Gentamycin and Amikacin were also placed on the MHA plate with the concentration 30 µg/ ml each from the Oxoid brand. The bacteria and dics were left to be dried about 10 minutes in room temperature. For the control, the Gram positive anti-bacterial agent is strictly for Amikacin and for Gram negative anti-bacterial agent is for Gentamycin (Arslan *et al.*, 2009). All the petri dishes were incubated at 30-35 °C for 24 hours. The inhibition zone that formed were measured in mm by using the measuring scale.

3.4 Antifungal Testing

This method was based on Srichana *et al.*, (2009). Each of the petri dish were distributed onto 20 ml of PDA agar. Circular block of the fungi from each stock culture about 5 mm in diameter were punched using a sterile tips and centrally placed onto the medium which is incorporated with the concentration of the chemical in the petri dish. About two replicates were maintained for each concenration and fungus. The culture were incubated at 30 °C for 4-5 days. The growth of each fungus will be measured at day 4 or 5. Using the means value, the percentage of inhibition was calculated by the formula:

$$\% \text{ Inhibition} = 100 - \frac{growth\ in\ treated}{growth\ in\ control}(100)$$

4.0 Result and Discussion

4.1 Antibacterial Test

4.1.1 Disc Diffusion

About seven thiourea complexes were tested on six different types of bacteria. In general the result showed that all of the thiourea compound complexes series produced an inhibition zone with the measurement range of 6.0 mm-14.0 mm as shown in table 4.1a-g. Similar results also reported in other studies shown by Arslan *et al*., (2009). In their studies, they reported that the synthesized thiourea compounds inhibit the growth of bacteria with the values ranging between 50 and 400 $\mu g/cm^3$. According to Gulkok *et al*., (2011), their study of thiourea on bacteria also gave positive result which exhibited a good inhibitory profile against bacteria. The zone of inhibition that formed was determined by measuring the diameter of zone which can be illustrated by Figure 4.1. The inhibition zone formed on MHA plates were illustrated in Figure 4.2.

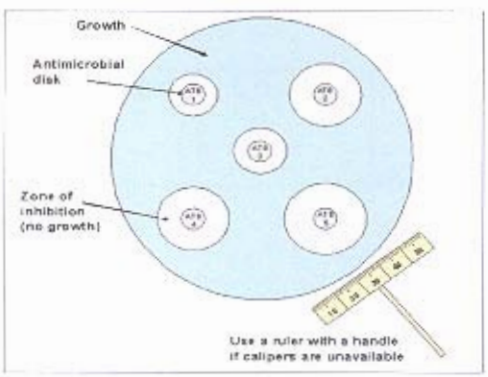

Figure 4.1: Pictures illustrates the measurement method of zone of inhibition.

Table 4.1a: Antibiotic susceptibility testing for Series 1 of thiourea complex in different concentration against 6 types of bacteria.

Series 1 (Toluidine + Ni Complex)

Concentration / Tested Bacteria	Zone of Inhibition (mm)					
	10.0 µg/ml	**5.0 µg/ml**	**2.5 µg/ml**	**1.25 µg/ml**	**0.625 µg/ml**	**Control: 30 µg/ml**
B. cereus	-	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	24.0
L. monocytogenes	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	19.0
S. aureus	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	22.0
S. entriditis	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	17.0
E. coli	7.0 ± 0.0	7.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	20.0
E. aerogenes	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	23.0

Table 4.1b: Antibiotic susceptibility testing for Series 2 of thiourea complex in different concentration against 6 types of bacteria.

Series 2 (Toulidine + Cu complex)

Concentration / Tested Bacteria	Zone of Inhibition (mm)					
	10.0 µg/ml	**5.0 µg/ml**	**2.5 µg/ml**	**1.25 µg/ml**	**0.625 µg/ml**	**Control: 30 µg/ml**
B. cereus	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	26.0
L. monocytogenes	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	16.0
S. aureus	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.5 ± 0.7	6.0 ± 0.0	21.0
S. entriditis	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	23.0
E. coli	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	17.0
E. aerogenes	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	23.0

Table 4.1c: Antibiotic susceptibility testing for Series 3 of thiourea complex in different concentration against 6 types of bacteria.

Series 3 (Anilline + Cu)

Concentration / Tested Bacteria	Zone of Inhibition (mm)					
	10.0 µg/ml	**5.0 µg/ml**	**2.5 µg/ml**	**1.25 µg/ml**	**0.625 µg/ml**	**Control: 30 µg/ml**
B. cereus	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	23.0
L. monocytogenes	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	20.0
S. aureus	7.0 ± 0.0	6.5 ± 0.7	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	24.0
S. entriditis	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	16.0
E. coli	7.0 ± 0.0	7.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	17.0
E. aerogenes	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	22.0